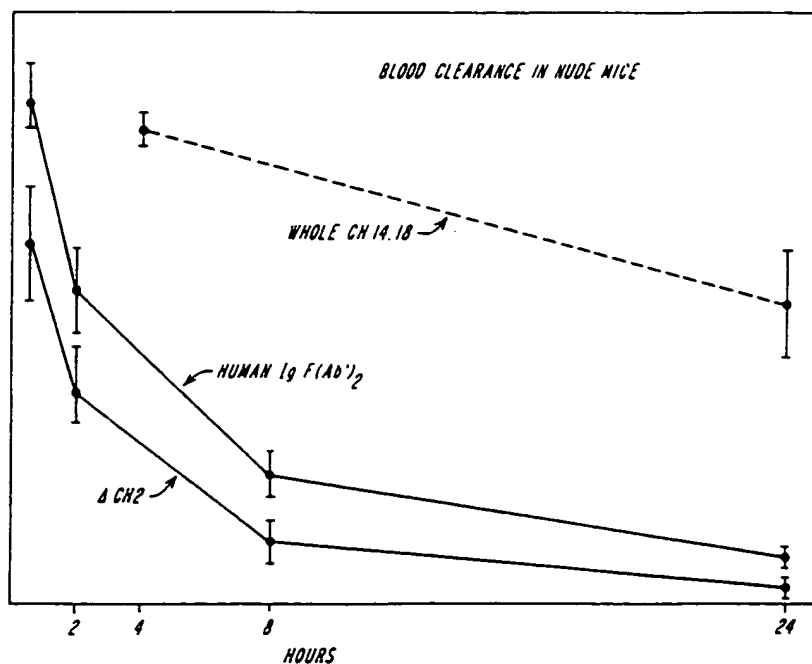




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12P 21/00, C07K 15/00 A61K 39/395	A1	(11) International Publication Number: WO 91/13166 (43) International Publication Date: 5 September 1991 (05.09.91)
(21) International Application Number: PCT/US91/00633 (22) International Filing Date: 30 January 1991 (30.01.91) (30) Priority data: 488,297 2 March 1990 (02.03.90) US (71) Applicant: ABBOTT BIOTECH, INC. [US/US]; 119 Fourth Avenue, Needham Heights, MA 02194 (US). (72) Inventor: GILLIES, Stephen, D. ; 245 Leavitt Street, Hingham, MA 02403 (US). (74) Agents: PITCHER, Edmund, R.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US) et al.		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: ANTIBODY CONSTRUCTS WITH ENHANCED BINDING AFFINITY



(57) Abstract

Disclosed is an antibody construct with enhanced binding activity specific for an epitope on an antigen. The construct includes an immunoglobulin binding region of predetermined specificity and having plural hypervariable regions homologous with a hypervariable region of a native antibody specific for the epitope. The construct further includes an immunoglobulin constant region consisting essentially of a CH1 domain, a hinge, and a CH3 domain and omitting the CH2 domain normally present in a native antibody. The immunoglobulin constant region enhances the binding activity of the construct at least two fold in comparison with the binding activity of a native antibody having the same specificity.

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## ANTIBODY CONSTRUCTS WITH ENHANCED BINDING AFFINITY

5 Background of the Invention

This invention relates to cancer imaging agents and cancer therapeutic agents, and more specifically, to the preparation of antibody  
10 constructs useful as imaging agents and/or cancer therapeutic agents. In particular, this invention relates to biosynthetic antibody constructs with enhanced antigen binding affinities and a short half life in vivo.

15

Systemic pharmacotherapy is a commonly used mode of therapy. Likewise is the administration of labelling agents such as radioisotopes for tumor or organ imaging. However, the administration of such  
20 drugs and radiolabels, by themselves, is risky in that the drugs usually are not target selective. Limiting the effects of chemotherapeutic drugs and radioisotopes used in the treatment of cancer are particularly difficult because these chemicals have  
25 the ability to interfere with the metabolic processes of most cells, especially those that are in a proliferative state. Therefore, the art frequently suggests coupling such drugs to an agent capable of targeting a particular tissue or cancerous cell  
30 type.

Antibody molecules have been conjugated to various drugs, enzymes, toxins, and radioisotopes (reviewed in Ghose and Blair (1978) J. Natl. Cancer

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Inst. 61:657-676). For example, antibodies have been successfully employed to image occult tumors not detected by traditional detection methods such as magnetic resonance imaging, x-ray analysis, and  
5 computed tomography (see Goldenberg (1988) Arch. Pathol. Lab. Med. 112:580-587).

However, a number of problems have been associated with the use of antibody molecules as  
10 targeting agents. Radiolabeled antibodies having a specificity for tumor antigen may show accretion rates of only 0.01% to 0.001% in the targeted tumor (Goldenberg, ibid.). In addition, there may be a high level of nonspecific binding of the intact  
15 antibody despite its specificity for a particular tissue-associated antigen. Some of this nonspecific binding has been attributed to the adherence of the antibody via its Fc portion, rather than via its binding domain. Furthermore, intact antibodies have  
20 a slow clearance rate relative to smaller molecules such as fragments of antibody molecules.

In an effort to alleviate these problems, the prior art suggests the use of antibody fragments  
25 which maintain their specificity and ability to bind antigen. Antibody fragments display more rapid specific targeting than intact antibodies. Wahl et al. (1983) J. Nuclear Med. 24:317-325). Currently, enzymatically produced F(ab')<sub>2</sub> fragments are most  
30 commonly used for such applications. These fragments lack the potentially troublesome Fc portion of the intact antibody molecule, and so demonstrate lower non-specific accumulation in the liver and spleen and a faster rate of clearance than intact antibodies.

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For example, several days after the administration of radiolabelled, intact antibody IgG, the level of circulating and nonspecifically bound radioactivity is high, and clearance occurs gradually over a period of four days or more (Goldenberg, ibid.). In comparison, the use of antibody fragments without Fc domains permits tumor imaging within 24-48 hours, thereby permitting the use of radioisotopes with short half lives, such as  $^{123}\text{I}$  and  $^{99\text{m}}\text{Tc}$  (Goldenberg, ibid.).

However, the use of  $\text{F(ab')}_2$  fragments is not without pitfalls. Besides being difficult to produce enzymatically, the binding properties of these fragments may be compromised (see, e.g., Wahl et al., ibid.). Therefore, what is needed is a targeting molecule having specificity, enhanced binding activity, minimal nonspecific binding abilities, and a shorter half-life in vivo.

Towards this end, monoclonal antibodies with increased antigen binding activities have been identified which have mutations in their constant regions (see, e.g., Pollock et al. (1988) Proc. Natl. Acad. Sci. USA 85:2298-2302). In addition, the ability to genetically engineer and re-express antibodies in transfected cells has made it possible to combine various portions of the immunoglobulin molecule as fusion proteins (Neuberger et al. (1984) Nature 312:614; Morrison et al. (1987) Ann. N.Y. Acad. Sci. 507:187; and Morrison et al. (1988) Clin. Chem. 34:1668). It is also possible to genetically encode immunoglobulin deletion mutants such as Fab (Horwitz et al. (1988) Proc. Natl. Acad. Sci. U.S.A.

85:8687) or F(ab')<sub>2</sub>-like fragments. In one study, an Fd' fragment, a truncated form of the immunoglobulin heavy chain, was expressed in *E. coli* (Cabilly et al. (1984) Proc. Natl. Acad. Sci. USA 81:3273-3277). In 5 another study, specific constant region domains of the human C<sub>γ</sub>3 H chain were either deleted or added in order to assess the effect on antibody synthesis and assembly (Morrison et al. (1987) Ann. N.Y. Acad. Sci. 507:1873).

10

Accordingly, it is an object of the present invention to provide antibodies which recognize tumor antigens, and would therefore be useful for the imaging and/or killing of tumors. It is also an 15 object of the present invention to provide antibody constructs useful for tumor imaging and/or cancer therapy which have reduced Fc receptor binding so that their non-specific accumulation in the spleen and other parts of the body is reduced. Additional 20 objects include the provision of antibody constructs which can bind quickly to tumor antigens and the provision of antibody constructs with shorter half lives than intact, whole antibody molecules.

### Summary of the Invention

It has been discovered that antibody constructs retaining their CH1 and CH3 domains, but  
5 lacking their CH2 domain, surprisingly have an enhanced binding affinity as well as a shorter half-life and an unexpectedly low level of non-specific binding relative to an intact antibody molecule with the same specificity. This finding has  
10 been exploited to develop the present invention which features antibody constructs having specificity for an epitope, present on various tumor antigens.

Native antibody molecules of the IgG and IgD  
15 classes are composed of two heavy (H) and two light (L) chains which are held together by covalent (disulfide) bonds and non-covalent interactions. The variable (V) domains at the amino termini of the two chains together form the antigen binding region. The  
20 first constant (C) domain of the H chain (CH1) interacts with the C region of the L chain through hydrophobic interactions and also through a disulfide bond. The next H-chain domain, adjacent to the hinge, is called CH2. This domain reportedly  
25 contains many of the effector functions of the antibody including the sequences responsible for complement fixation and Fc receptor-mediated antibody-dependent cellular cytotoxicity (ADCC), and is the sole N-linked glycosylation site in human C<sub>γ</sub>1  
30 chains. The CH3 domain at the carboxy terminus of the H-chain is thought to play a major role in the assembly of H chains.

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The antibody constructs of the present invention include a first portion having an immunoglobulin binding region of predetermined specificity. This binding region has plural  
5 hypervariable regions homologous with hypervariable regions of a native antibody specific for the same epitope. The antibody constructs further include a second portion consisting essentially of a CH1 domain and a hinge whose C terminus is linked to the N  
10 terminus of a CH3 domain, i.e., a hybrid constant domain which omits the CH2 domain normally present in a native antibody. Antibody constructs with the CH2 domain deleted are referred to hereinafter as  $\Delta$ CH2 constructs.  $\Delta$ CH2 constructs are characterized by  
15 enhanced binding activity, at least twofold greater than the binding activity of an antibody construct with the same specificity but having an intact constant domain.

20 In one preferred embodiment, the binding region of the  $\Delta$ CH2 antibody construct includes the binding region of a nonhuman immunoglobulin such as a murine immunoglobulin. In one aspect of the invention, the immunoglobulin binding region is one  
25 which has a predetermined specificity for mucin such as the binding region of monoclonal antibody B72.3, or has a predetermined specificity for the G<sub>D2</sub> ganglioside such as the binding region of monoclonal antibody 14.18. In another embodiment of the  
30 invention, the immunoglobulin binding region has a predetermined specificity for a melanoma-specific proteoglycan. The preferred altered constant region includes a human immunoglobulin constant domain.



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The antibody construct typically includes an immunoglobulin heavy chain disulfide-linked to an immunoglobulin light chain.

5           The antibody construct further may include a third portion bonded to the carboxy terminus of the the second portion comprising a tumoricidal protein. This third protein portion preferably is lymphotoxin, interleukin-2, epidermal growth factor (EGF), active  
10 analogs thereof, or active fragments thereof. In a preferred embodiment of the invention, the third portion is peptide bonded via an endopeptidase-cleavable amino acid residue, such as lysine, to the carboxy terminus of the CH3 domain of the second  
15 region. Alternatively, the third portion may be linked to the second portion by chemical crosslinking, for example.

In yet another embodiment, for use as an  
20 imaging agent, the  $\Delta$ CH2 antibody construct includes a radioactive label attached thereto.

Brief Description of the Drawing

The foregoing and other objects of the present invention, the various features thereof, as well as the inventions thereof may be more fully understood from the following description when read together with the accompanying drawings in which:

FIG. 1 is a schematic representation showing the construction of a  $\Delta$ CH2 deletion mutant form of the human Cyl gene: (A) is a map of the HindIII to PvuII fragment containing the genomic Cyl gene segment and restriction sites; (B) is the  $\Delta$ CH2 deleted gene missing the AflIII to AvaII fragment (the CH2 exon) after restriction and ligation; (C) shows the SmaI site engineered in the CH3 gene; (D) is a map of a SmaI to PvuII linker which is used to attach the (E) PvuII to XhoI fragment containing an IL2 gene; and (F) is the remainder of the vector including an XhoI site and polyA region;

FIG. 2 is a graphic representation of the antigen binding activity of various  $\Delta$ CH2 chimeric constructs: (A) the  $\Delta$ CH2 chl4.18 antibody construct; (B) the  $\Delta$ CH2 chimeric B72.3 (chB72.3) antibody construct;

FIG. 3 is a graphic representation of the competitive antigen binding analyses of the intact chl4.12 antibody and the  $\Delta$ CH2 chl4.18 antibody constructs after (A) 2 hours incubation at 37°C; and (B) 18 hours incubation at 4°C;

FIG. 4 is a graphic representation of the antibody-dependent cellular cytotoxicity (ADCC) of intact antibodies and  $\Delta$ CH2 antibody constructs;

5           FIG. 5 is a graphic representation of the complement-dependent cytotoxicity (CDC) of intact antibodies and  $\Delta$ CH2 antibody constructs;

10           FIG. 6 is a graphic representation of the biodistribution of (A) intact ch14.18 antibody and (B) the ch14.18  $\Delta$ CH2 antibody construct over time within athymic nude mice bearing M21 xenographic tumors;

15           FIG. 7 is a graphic representation of the biodistribution of F(ab')<sub>2</sub> fragments and the  $\Delta$ CH2 antibody construct within nude mice bearing M21 xenographic tumors; and

20           FIG. 8 is a graphic representation of the clearance of the  $\Delta$ CH2 antibody construct, intact antibody, and F(ab')<sub>2</sub> fragments from the circulation of athymic nude mice bearing M21 xenographic tumors.

25

Description of the Invention

The present invention features truncated antibody constructs having specificity for a tumor-associated or other antigen, and demonstrating the in vivo properties of an enhanced binding activity, a relatively short half-life, and a relatively low level of nonspecific binding. These ACH2 constructs are well suited as targeting agents for tumor imaging and systemic pharmacotherapeutic treatment.

The invention provides antibody constructs with binding specificity for an epitope on a human tumor antigen. The specificity of the antibody preferably is for a tumor antigen which enables the selective targeting of that tumor, i.e., the construct binds to a unique marker for the tumor. Unfortunately, only a few antigen specific markers for human cancers have been identified. However, human tumor cells do have tumor-associated antigens which are present in smaller quantities in certain normal cells such as embryonic cells. Such antigens include alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA). Antibody constructs which recognize these or other tumor-associated antigens such as mucin (B72.3 antibody) and the disialoganglioside GD<sub>2</sub> (14.18 antibody) are included in this invention.

The ACH2 constructs of the present invention can be prepared by genetic manipulation of the DNA which encodes the various domains of an antibody (see, e.g., Neuberger et al. (1984) Nature 312:604; Morrison et al. (1987) Ann. N.Y. Acad. Sci. 507:187;

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Morrison et al. (1988) Clin. Chem. 34:1668; Horwitz et al. (1988) Proc. Natl. Acad. Sci. 85:8678).

In order to reduce immunogenicity of the  $\Delta$ CH2 constructs in humans, the  $\Delta$ CH2 constructs were designed to have a minimal amount of nonhuman protein. This may be accomplished by creating chimeric antibody molecules having at least human constant regions and mammalian, e.g., mouse, variable 10 regions.

The production of recombinant chimeric antibodies with predetermined specificity has typically involved the use of cloned genomic DNA 15 fragments. For example, the genomic DNA sequences encoding H and L chains can be cloned in their rearranged forms (i.e., in the DNA sequence that results from recombination events during B Cell maturation). As such, these genomic sequences 20 contain the information necessary for their expression, (i.e. the 5' untranslated sequences, promoter, enhancer, protein coding region, and donor splice site). The donor splice signals at the 3' end of the V gene segments are compatible with the splice 25 acceptor signals at the 5' end of the Ig regions of other species. That is, the splice product between the two maintains the correct reading frame. For example, when a murine V and a human C<sub>k</sub> segment are joined and transfected into the appropriate host cell 30 type, the primary transcript is correctly spliced and results in a mature messenger RNA (mRNA) molecule with an open reading frame through both the V and C regions.

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A preferred method of producing the chimeric ACH2 constructs involves the preparation of an immunoglobulin constant region-encoding cassette including a DNA sequence which enables the splicing of that immunoglobulin constant region-encoding segment to an immunoglobulin variable region-encoding DNA segment having a compatible splice sequence, thereby allowing subsequent transcription and translation of an immunoglobulin heavy or light chain. This method is described in co-pending patent application serial number 409,889 entitled "Method of Producing Fusion Proteins", filed September 20, 1989, herein incorporated as reference.

Briefly, a DNA cassette is prepared by reconstructing the 3' end of a splice donor site, and its attachment to the 3' end of a DNA sequence or exon encoding a variable (V) region. The cassette is transfected with expressable DNA (structural gene) for a constant (C) region gene having a compatible splice acceptor site at its 5' end.

During the sequence of events leading up to expression in the transfected cell of the fusion protein, mRNA derived from the two DNA sequences are spliced to produce a mature mRNA having a 5' end encoding a complete  $V_H$  or  $V_L$  and a 3' end encoding a human  $C_H$  or  $C_L$  domain. The resulting single chain polypeptide is a fusion of the V region and the constant domain encoded by the exon of the structural gene. In addition, a tumoricidal agent-encoding cassette may be spliced to an Ig C region which, in turn is spliced to an immunoglobulin V region,

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resulting in a "magic bullet"-type pharmacotherapeutic agent.

The cDNA encodes a H or L chain V region  
5 (most likely non-human, e.g., murine) specific for a tumor antigen while the C region exon(s) encode the H or L chain C region (CH1, CH2, and CH3 domains) of another (most likely human) Ig species. Useful V regions include the V<sub>H</sub> and V<sub>L</sub> domains of murine  
10 monoclonal antibody 14.18 (Mujoo et al. (1987) Cancer Res. 47: 1098-1104) which recognizes the disialoganglioside G<sub>D2</sub> on the surface of many neuroblastoma, melanoma, glioma, and small lung carcinoma lines and tissues. Other useful V regions  
15 include the V<sub>H</sub> and V<sub>L</sub> domains of murine monoclonal antibody B72.3 (Johnson et al. (1986) Cancer Res. 46:850) which recognize a mucin-like structure on many breast and colon carcinomas. These V region can be combined with various C regions such as that of  
20 the Ig human gamma (H) and kappa (L) chains. Alternatively, the V region may be of human origin. Expression of H and L constructs in a single competent host cell results in production of intact chimeric immunoglobulins having a desired specificity  
25 and, for example, an intact human constant region.

To produce the  $\Delta$ CH2 construct, a V region cassette is constructed and placed in an appropriate vector, together with a C region-encoding DNA  
30 sequence. This C region specifically lacks the CH2 domain and is designed as shown in FIG. 1. FIG. 1A shows a map of the HindIII to PvuII fragment containing the human genomic C $\gamma$ 1 gene segment. FIG. 1B shows the DNA encoding the C region of the  $\Delta$ CH2

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construct after restriction and ligation of the two fragments with a synthetic linker (to join the AflIII and AvaII ends).

5            Vectors encoding this mutant C region are transfected into an appropriate host capable of expressing the vectors. Upon co-expression of the two DNAs, the host cell nuclear enzymes produce mRNA by implementing the normal splicing events, resulting  
10 in an mRNA encoding a fused protein. In the case of a chimeric binding protein, the mRNA may encode (5' to 3') a  $V_H$  or  $V_L$  domain, which may be identical to the native sequence up to the VC junction, attached directly to another polypeptide such as at least a  
15 portion of at least one C region domain, which for example, may comprise human sequences. The 3' half of the donor splice site (and any nucleotides downstream) and the 5' half of the acceptor splice site (and any nucleotides upstream) are removed as an  
20 intron, resulting in an mRNA encoding the properly fused protein.

Normally both exons are placed on the same vector under control of a single regulating  
25 sequence. Also, it is preferred to coexpress both L and H chain constructs so that the host cell secretes an intact fusion protein. The method requires use of a host cell having the enzymes which recognize the DNA splice signals and effect proper splicing.

30

Particular vector construction, host cell selection, transformation, and methods of expression do not, per se, constitute an aspect of the invention, but can be selected and implemented by



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skilled workers based on personal preference and convenience. Techniques adaptable for use in the invention are disclosed, for example, in Current Protocols in Molecular Biology (Greene Publishing Associates, 430 Fourth Street, Brooklyn, N.Y., 1989). Useful vectors include any number of known plasmids which contain the correct signals for transcription and translation of the genes of interest. Enhancer elements may be present, and additional signals for polyadenylation and splicing must be present in cases where they are not provided by the gene itself. For example, all of the signals for the expression of functionally rearranged Ig genes are present on a continuous stretch of DNA and include the transcription promoter, the splicing signals for excision of the intron sequences and the polyadenylation and termination sites. Additional information that must be provided by the vector is a selectable marker gene. This gene must also contain the signals for expression of the selectable phenotype (usually resistance to the lethal effect of a toxic drug such as methotrexate, for example). Therefore, if the vector encodes both the first and second polypeptides, it is necessary that it provide the sequence information for three separate transcription units in a limited amount of space.

The recombinant cassette-containing vector is transfected into an appropriate host cell. The choice of host cell line, in addition to the criterion noted above, is based on its ability to grow in a growth media, preferably one that is commercially available and serum-free as well as its ease of selectivity after transformation. For the

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production of chimeric antibodies, useful host cells include myelomas or hybridomas such as, for example, the murine non-Ig-producing Sp2/0 Ag 14 hybridoma cell line, yeast, bacteria, and plant cells. Useful 5 host cells are widely available in repositories and from commercial sources and may be isolated readily from natural sources by those skilled in the art.

One method for introducing recombinant DNA 10 into cells is electroporation (see, e.g., Potter et al. (1984) Proc. Natl. Acad. Sci. USA 81:7161-7165), which requires specialized equipment and the availability of highly purified DNA. However, many different lines can be transformed using this method 15 if conditions are optimized for the specific cell type.

Another transfection method is protoplast (spheroplast) fusion (see, e.g., Sandri-Goldin et al. 20 (1981) Molec. Cell. Biol. 1:743-752). Bacteria harboring the recombinant plasmid of interest are fused to the lymphoid cells with a chemical agent, generally 45-50% polyethylene glycol in a buffered, isotonic solution. This method is simple and does 25 not require extensive purification of plasmid DNA. In addition, very high transformation frequencies can be obtained, and the time for obtaining highly productive transfected cell clones is reduced because this transfection method is likely to give 30 transfectants containing multiple copies.

Cells which are successfully transformed with the vector must then be isolated from those which are not. Many methods are available for the

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selection of transfected cells. For example, the guanine phosphoribosyl transferase (gpt) and neomycin (neo) resistance markers may be used for selection purposes in lymphoid cells. The gene encoding the  
5 marker would be included on the V-region encoding vector. The resistant form of dihydrofolate reductase (DHFR) can also be used for the selection of hybridoma cell transformants as well as for subsequent amplification of the marker and flanking  
10 product genes.

The transfected cell is then cultured to express the polypeptide encoded by the cassette. Culturing may be in vitro, or in the case of  
15 recombinant antibodies, may be accomplished by employing other strategies such as in vivo culturing in ascites fluid.

The CH2 domain of the human C $\gamma$ 1 heavy chain  
20 which is deleted from the intact antibody molecule includes the following sequences: the sequence conferring long half-life; the sequence responsible for binding the Fc receptor on effector cells; the sequence to which the single N-linked carbohydrate  
25 chain is attached; and the sequence to which the first complement component, C1q, binds.

To determine to what extent these sequences are necessary for various antibody functions, the  
30  $\Delta$ CH2 constructs were examined closely. When the supernatants of cells expressing the intact and  $\Delta$ CH2 antibodies were first quantitated by measuring associated human  $\kappa$  chain to normalize the data to the number of antigen binding domains (antibody molecules

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contain one  $\kappa$  light chain per antigen binding site), and then compared in a direct antigen binding assay, the  $\Delta$ CH2 constructs demonstrated much higher activity, as shown in FIG. 2. The chl4.18  $\Delta$ CH2 construct was assayed on G<sub>D</sub>2-coated plates, while the chB72.3  $\Delta$ CH2 construct was assayed on mucin-coated plates.

The antigen binding activity of the  $\Delta$ CH2 construct was tested further in a competitive binding format in order to rule out the possibility of non-specific interactions with the ELISA plates. As seen in FIG. 3, the chl4.18  $\Delta$ CH2 construct competes much more efficiently with the labeled intact chl4.18 antibody for antigen than the normal antibody competes with itself. When the length of the binding assay is extended from 2 hours (FIG. 3A) to 18 hours (FIG. 3B), the difference in binding is not as dramatic, suggesting that the rate of antigen binding and not the overall affinity is increased by the removal of the CH2 domain.

It is hypothesized that the increased antigen binding of  $\Delta$ CH2 antibody constructs reflects conformational changes in the antibody molecule. By removing the CH2 domain, the Fab region (including both V region, C<sub>L</sub> and CH1 sequences) is no longer restricted by interactions with portions of the CH2 domain. Removal of these inter-domain interactions appears to increase the rate of association with antigen. In addition, the removal of the carbohydrate moiety of the CH2 domain may reduce steric interactions between the antibody and the antigen.

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That the effector functions attributed to the CH2 domain are no longer retained in the  $\Delta$ CH2 antibody can be confirmed by ADCC assay using the intact ch14.18 antibody as a basis for comparison.

5 The results of a typical ADCC assay, which measures the ability of unfractionated peripheral blood leukocytes (PBLs) to lyse melanoma target cells as a function of the amount of added antibody, are shown in FIG. 4. Here, the specific lysis of M21 human

10 melanoma cells in the presence of the indicated concentrations of ch14.18 antibody or  $\Delta$ CH2 construct is shown for an effector to target ratio of 100:1. The average of triplicate samples is shown for each data point. The intact ch14.18 antibody is a very

15 potent mediator of ADCC, even at levels as low as 1 ng/ml. Surprisingly, the  $\Delta$ CH2 construct exhibits a very small amount of ADCC activity.

The loss of ADCC activity also reflects a

20 loss of the ability to bind to Fc receptors. Since the site for binding of the high-affinity receptor (FCR) has been mapped to the CH2 domain, adjacent to the hinge, it is not surprising that the  $\Delta$ CH2 mutant had greatly reduced activity. For the radioactive

25 imaging of tumors, it is important that Fc receptor binding be reduced so that there is minimal nonspecific accumulation in the spleen, where many Fc-receptor-bearing cells localize. The  $\Delta$ CH2 construct here described is therefore useful in this

30 regard.

As expected, the  $\Delta$ CH2 construct also was found to have a reduced ability to mediate the complement lysis of melanoma target cells. FIG. 5

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shows that the  $\Delta$ CH2 mutant had no measurable ability to lyse M21 cells in the presence of human complement relative to the very potent chl4.18 antibody.

5           Anti-tumor  $\Delta$ CH2 antibodies, having the ability to specifically and rapidly localize to the tumor, also serve as ideal vehicles for the delivery of therapeutic agents. For example, as shown in FIG. 1(C)-1(E), the DNA sequence encoding human  
10 interleukin-2 (IL2) may be attached to the carboxy terminus of an anti-tumor  $\Delta$ CH2 construct, thereby resulting in the expression of a  $\Delta$ CH2/IL2 fusion protein that serves to deliver IL2 to the tumor site. The local accumulation of IL2 activates  
15 T-cells at the tumor site, thereby hastening its destruction. Although the half-life of  $\Delta$ CH2 antibodies is shorter than whole antibodies, the  $\Delta$ CH2/IL2 fusion protein should be much longer than the half-life of IL2.

20           In another example, the anti-tumor cytokine lymphotoxin or tumor necrosis factor  $\beta$  (TNF  $\beta$ ), could be fused to the carboxy terminus of a  $\Delta$ CH2 antibody. This fusion protein also serves to specifically  
25 localize this cytotoxic protein to a tumor, thus reducing its potential adverse systemic effects. Likewise, other toxic proteins could be fused to  $\Delta$ CH2 antibodies. These include protein toxins such as ricin, diphtheria toxin, and Pseudomona exotoxin or  
30 those portions of the whole toxin molecule that is responsible for toxicity, i.e. the ADP-ribosylating enzyme activity that leads to the inactivation of mammalian cell elongation factor 2.

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The specificity and half-life of various antibody constructs in vivo can be characterized by performing biodistribution studies. Intact,  $\Delta$ CH2, or  $F(ab')_2$  are injected into M21 xenographic tumor-bearing animals, and the amount of construct in various body organs and tissues are monitored over time or at a particular time. FIG. 6 and TABLE 1 show the biodistribution of (A) intact chl4.18 antibody and (B) a  $\Delta$ CH2 construct with time, and demonstrates that the latter targets quickly (within 4 hours) and specifically to the tumor.

TABLE 1

15				<u>Time</u>	
	<u>Antibody</u>	<u>Sample</u>	<u>4 hr.</u>	<u>24 hr.</u>	<u>96 hr.</u>
	(A) chl4.18				
		tumor/blood	0.18	0.56	1.08
		liver/blood	0.79	2.02	4.55
20	(B) ΔCH2				
		tumor/blood	0.86	2.20	1.19
		liver/blood	2.98	7.37	2.28

FIG. 7 shows the biodistribution of human Ig  $F(ab')_2$  fragments and chl4.18  $\Delta$ CH2 constructs 24 hours after injection, and demonstrates the specificity of targeting of the  $\Delta$ CH2 constructs.

The half-life of the  $\Delta$ CH2 antibody constructs in circulation may be determined by injecting them into the circulation of tumor-bearing animals and monitoring their presence in the blood

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with time. FIG. 8 demonstrates the short half-life of  $\Delta$ CH2 constructs relative to F(ab')<sub>2</sub> fragments and intact antibodies in the circulation of nude mice bearing M21 xenographic tumors.

5

Because the  $\Delta$ CH2 antibody constructs are able to bind quickly to a tumor epitope with relatively little non-specific binding to other tissues, they can be used to target various  
10 therapeutic agents to tumors. Particularly useful therapeutic agents include those having direct or indirect tumoricidal activity such as interleukin-2, lymphotoxin, or epidermal growth factor. For example, EGF is fused to an antibody that binds to  
15 and activates a cytotoxic T-cell, thus cross-linking the T cell and EGF receptor-bearing cell. Fragments and biosynthetic analogs of these proteins having tumoricidal activity also are useful. These proteins may be chemically linked to the CH3 domain via, for  
20 example, various cross-linking agents known in the art, or, as disclosed herein, may be peptide-bonded to the carboxy terminus of the CH3 domain. For example, the vectors constructed to encode the  $\Delta$ CH2 constructs may further encode the tumoricidal  
25 protein.

The increased binding activity of the  $\Delta$ CH2 construct, together with its shorter half-life in vivo, and its low level of nonspecific binding make  
30 this molecule extremely useful in the radioactive imaging and/or treatment of tumors. The constructs may be labeled with, for example, radionuclides of low energy such as iodine-123, indium-111, or technetium-99m, or with any radioactive isotopes that



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will not change the binding characteristics of the construct when attached thereto, such as iodine-125. When labelled with a suitable radioisotope the ΔCH2 antibody constructs enable detection of the locus of  
5 a tumor-related antigen by external detection of emitted radiation. Radioactive gamma emitters are preferred, although in some circumstances positron emitters, such as  $^{64}\text{Cu}$ ,  $^{11}\text{C}$ , and  $^{15}\text{O}$  may be used. It is also contemplated that the ΔCH2 antibody  
10 constructs may be labelled with a paramagnetic substance such as gadolinium so that concentrations of the reagent localized about a tumor may be imaged by nuclear magnetic resonance imaging techniques.

15 Targeting of a construct with a tumoricidal agent and/or radionuclide may require a higher concentration of construct having a longer half-life than does imaging. The level of radioactivity required for imaging depends in part on the ability  
20 of the antibody construct to selectively label tumor relative to surrounding tissue, the size of the tumor, and the distance of the tumor from the injection site.

25 The ΔCH2 antibody constructs of the invention can be labelled with such agents using conventional techniques used to label other proteins known to those skilled in the art. One currently preferred technique is to express a ΔCH2 antibody  
30 construct, covalently linked on either its 3' or 5' end to a lysine rich polypeptide sequence comprising, for example, 2-20 residues, to serve as a site of attachment for the remotely detectable moieties

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discussed above. The gamma or positron emitting ions are preferably attached to these linkers by ionic interaction or chelation with the free amine groups on the lysine residues.

5

For example, diethylenetriamine penta acetic acid (DTPA) may be used to label with Tc-99 or In-111. In or I isotopes may be attached using the oxine salt (8-hydroxy quinoline). Iodogen (1, 3, 4,  
10 6 tetrachloro - 3 $\alpha$ , 6 $\alpha$  diphenylglucoluril - Pierce Chem. Co., Roskville, Ill) may be used for labeling with <sup>125</sup>I. (See, e.g., Thakur et al. Thromb. Res. 9:345 (1976); McFarlane, J. Clin. Invest. 42:346 (1963); Knight et al., Radiopharmaceuticals, N.Y.  
15 Soc. of Nuclear Medicine, 1975, pp. 149; and Harwig et al., Int. J. Appl. Radiat. Isot. 27:5, 1976.) Other methods are known to those skilled in the art.

In view of the foregoing, it will be  
20 apparent that other types of known reagents can be linked using conventional techniques to  $\Delta$ CH2 antibody constructs. Examples include therapeutic drugs which promote healings such as calcitonin, epidermal growth factor, tumor growth factor alpha and beta, platelet  
25 derived growth factor, insulin-like growth factor 1 (Somatomedin-C), connective tissue activating peptide, and human collagenase inhibitor.

The dosage and means of administration of  
30 the family of  $\Delta$ CH2 antibody constructs produced in accordance with the invention will necessarily depend on the nature of the drug involved, the degree if any that its bioactivity is reduced when it is present as a conjugate, the immunological tolerance of specific

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patients, and the nature of the diseased condition in question. When the polypeptide is linked to an imaging agent, it should be administered in an amount sufficient to be detected by scintillation scanning  
5 or other external radiation detection means capable of detecting the localized radiation present in the tumor, such as autoradiography. In general, about 5-20 microcuries should be administered; most preferably about 10 microcuries. The actual amount  
10 depends on the location of the tumor-associated antigen as is well known in the art. Additional radioactive peptide may be injected if necessary up to the amounts limited by prevalent standards of safety.

15

Tumors may be imaged or treated by systemic administration of the  $\Delta$ CH2 construct via intravenous injection. Their preferred dosage is in the range of from about 0.01 to 10 mg per kg body weight.

20

The invention will be further understood from the following, non-limiting examples.

#### EXAMPLES

25

##### 1. Plasmid Construction

The human Cyl gene, subcloned in pBR322 as a HindIII to PvuII fragment (Gillies et al. (1989) J.  
30 Immuno. Meth. 125:191-202) was used for the construction of the H chain mutants. The deletion mutant lacking the CH2 domain ( $\Delta$ CH2) was made by joining the HindIII to AflIII fragment (containing the CH1 and hinge exons) to an AvaII to PvuII

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fragment (containing the CH3 exon) using a synthetic double-stranded oligonucleotide fragment. The  $\Delta$ CH2 construct was checked by DNA sequence analysis and then introduced into the chimeric antibody expression vector pdHL2-VCy1C $\kappa$  (Gillies et al. *ibid.*) containing the V regions of the mouse monoclonal antibody 14.18 (Mujoo et al. (1987) Cancer Res. 47:1908).

A  $\Delta$ CH2-IL2 fusion construct was constructed by first introducing a SmaI site by mutation near the carboxy terminus of the  $\Delta$ CH2 human IgG1 gene (FIG. 1(C)). The mutation changes nucleotide 2373 (according to the numbering in the Huck et al., (1986) Nucl. Acids. Res. 14:1779-1789) from a T to a C in the wobble position of a serine codon and thus does not change the amino acid sequence. A synthetic DNA fragment was used to link the mature IL2 protein sequence to this SmaI site (FIG. 1D-1E). The sequence includes, from 5' to 3', the SmaI site, the remaining sequence of the human gamma 1 gene (three additional amino acids) fused directly to the first amino acid (alanine) of the mature IL2 protein sequence, and the amino terminal sequence of IL2 up to the unique PvuII site. The remaining IL2 sequence was then joined at the PvuII site and extends through the coding sequence to a unique XhoI site located 3' of the translational stop signal. The XhoI end of the joined DNA was ligated to a vector containing the 3' untranslated region and polyA addition site of the mouse  $\kappa$  constant region (FIG. 1F). The resulting construct encodes a  $\Delta$ CH2 human gamma 1 chain to which IL2 is attached at the carboxy terminus.

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Transfection and drug selection were carried out essentially as described in Gillies et al. (Bio/Technol. (1989) 7:799-804), herein incorporated as reference. Briefly, the non-Ig-producing murine hybridoma line, Sp2/0 Ag14, was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Plasmids were introduced into cells by protoplast fusion essentially as described in Gillies et al., (1983) Cell 33: 717-728). Transfectants were selected in growth medium containing methotrexate at an initial concentration of 0.1  $\mu$ M. MTX-resistant clones were tested for the secretion of human antibody by ELISA assay. Microtiter plates were coated with goat anti-human IgG (H and L specific-Jackson Laboratories) and incubated with conditioned medium from transfected cells. Goat anti-human IgG (Fc-specific-Jackson Laboratories), conjugated to horseradish peroxidase, was used for detection of human antibody. The clones that produced the greatest amount of antibody were cultured in medium containing increasing concentrations of MTX (from 0.1  $\mu$ M to 1  $\mu$ M and after adaptation, to 5  $\mu$ M and finally 10  $\mu$ M MTX). After a few passages in 10  $\mu$ M MTX, cells were subcloned by limiting dilution.

### 3. Purification of Proteins

The chl4.18 intact antibodies were purified by binding to and elution from protein A Sepharose (Repligen). The  $\Delta$ CH2 mutant antibody was purified by immunoaffinity chromatography on an anti-human  $\kappa$  monoclonal antibody-Sepharose column. Both proteins were >90% pure when analyzed by SDS-PAGE or HPLC.

#### 4. Antigen Binding Assays

Direct antigen binding assays as well as competitive binding assays were performed with the 5 disialoganglioside G<sub>D2</sub> antigen (14.18 antibody) or submaxillary gland mucin (Sigma) (B72.3 antibody)-coated microtiter plates as described in Gillies et al. (1990, ibid.). In both cases, the secondary (detecting) antibody was a goat anti-human IgG, 10 Fc-specific polyclonal antiserum (Jackson ImmunoResearch), labeled with horse radish peroxidase (HRP). Briefly, the G<sub>D2</sub>-coated plates were first blocked with 5% bovine serum albumin (BSA) and 5% goat serum in PBS for 2 hr at 37°C. Unlabelled goat 15 antibodies were diluted in assay buffer (1% BSA, 1% goat serum in PBS) and 25 µl was added to each well. After incubation at 37°C for 4 to 5 hours, 25 µl of HRP-conjugated 14.18 chimeric antibody (6.3 ng) was added to each well. Plates were covered and 20 incubated overnight at 4°C, washed six times with PBS, and developed with O-phenylenediamine (OPD) substrate.

#### 5. Cytotoxicity Assays

25

ADCC assays were carried out using <sup>51</sup>Cr-labeled M21 human melanoma target cells or any cell line which expresses G<sub>D2</sub>. A fixed number of labeled targets (2 x 10<sup>5</sup> cells/ml in 50 µL) and varying 30 concentrations of human effector cells (peripheral blood leukocytes from normal donors) in 50 µL were mixed with 100 µL of diluted chimeric antibodies in round-bottom microtiter plates. Following a 4 hr incubation at 37°C, the plates were centrifuged, and

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100  $\mu$ L samples were removed for counting in a LKB model 1272 gamma counter.

Complement-mediated lysis of labeled M21 melanoma cells was carried out by mixing 50  $\mu$ L of cells ( $2 \times 10^5$  cells/mL) with an equal volume of each antibody dilution. After a 15 min incubation at 37°C, 100  $\mu$ L of human complement (1:4 dilution of fresh human serum) was added to each well. Plates were incubated for an additional hour at 37°C, and the amount of released  $^{51}\text{Cr}$  was determined by centrifuging the plates and counting 100  $\mu$ L aliquots of the supernatants. The percentage of cytotoxicity was determined as:

15

$$\frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100$$

## 20 6. Biodistribution and Blood Clearance Studies

8 to 10 weeks old female athymic mice (nu/nu) (the National Cancer Institute, Bethesda, MD) were injected subcutaneously with  $2 \times 10^6$  M21 tumor cells. Tumors of 50-150 mg weight grew within 10 days. At this time, the animals received i.v. injections into the lateral tail vein of 25  $\mu$ g and 3-4  $\mu$ Ci  $^{125}\text{I}$ -labeled antibody. At designated time points after injection, groups of 3 animals were anesthetized with halothane, and blood samples were obtained by retro-orbital bleeding. For biodistribution of radiolabeled antibodies, groups of 3 animals were sacrificed at various time points after injection. Tumors and major organs (heart,

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skin, muscle, bone, lung, liver, spleen, thyroid, kidney, and intestine) were removed and weighed. All tissue samples were assayed in a gamma counter for  $^{125}\text{I}$  activity. The results were calculated as 5 percent of injected dose per g tissue and as localization ratios (cpm/g tumor : cpm/g tissue).

#### 7. Radioactive Labeling

10 Ch14.18, ch14.18-ACH2, and F(ab')<sub>2</sub> fragments of human IgG were labeled with  $^{125}\text{I}$ . Briefly, 500  $\mu\text{g}$  antibody was incubated for 25 min on ice with 0.5 mCi  $^{125}\text{I}$  (100 mCi or 3.75 GBq/ml, Amersham Corp.,  
15 Arlington Heights, IL) in polystyrene tubes coated with 100  $\mu\text{g}$  Iodo-Gen reagent (Pierce Chemical Co., Rockford, IL). Unincorporated  $^{125}\text{I}$  was removed by gel filtration on PD10 columns (Pharmacia Fine Chemicals, Piscataway, NJ). Specific activity was typically 1.5 - 0.5 nCi/ng antibody.

20

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore considered to be in all  
25 respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are  
30 therefore intended to be embraced therein.

What is claimed is:



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1. An antibody construct specific for an epitope on an antigen, said construct comprising:
  - 5 (a) an immunoglobulin binding region of predetermined specificity comprising hypervariable regions homologous with a hypervariable region of a native antibody specific for said epitope; and
  - 10 (b) an immunoglobulin constant region consisting essentially of a CH1 domain, a hinge, and a CH3 domain and omitting the CH2 domain normally present in a native antibody,
  - 15 said constant region enhancing the binding activity of said antibody construct at least two fold in comparison with the binding activity of said native antibody.
- 20 2. The construct of claim 1 wherein said binding region comprises a binding region of a nonhuman immunoglobulin molecule.
3. The construct of claim 2 wherein said  
25 binding region comprises a binding region of a murine immunoglobulin molecule.
4. The construct of claim 1 wherein said  
30 binding region has a specificity for mucin.
5. The construct of claim 1 wherein said binding region has a specificity for G<sub>D2</sub> ganglioside.

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6. The construct of claim 1 wherein said binding region has a specificity for a melanoma-specific proteoglycan.
- 5 7. The construct of claim 1 wherein said constant region comprises a human immunoglobulin constant domain.
8. The construct of claim 1 comprising an  
10 immunoglobulin heavy chain disulfide-linked to an immunoglobulin light chain.
9. The construct of claim 1 further comprising,  
15 attached to the carboxy terminus of said constant region, a protein having tumoricidal activity.
10. The construct of claim 9 wherein said protein is peptide bonded via an endopeptidase-cleavable amino acid sequence to the carboxy terminus  
20 of said CH3 domain.
11. The antibody construct of claim 10 wherein said protein is peptide bonded via a lysine residue to the carboxy terminus of said CH3 domain.  
25
12. The construct of claim 9 wherein said protein comprises a tumoricidal protein selected from the group consisting of lymphotoxin, active analogs thereof, and active fragments thereof.  
30
13. The construct of claim 9 wherein said protein comprises a tumoricidal protein selected from the group consisting of interleukin-2, active analogs thereof, and active fragments thereof.

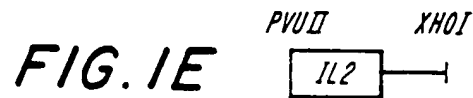
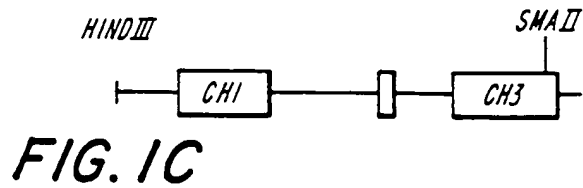
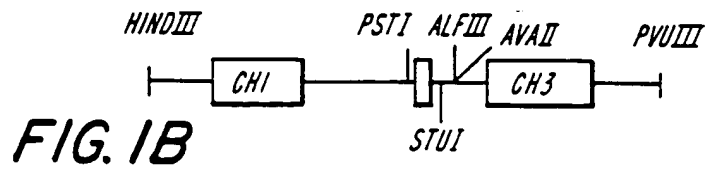
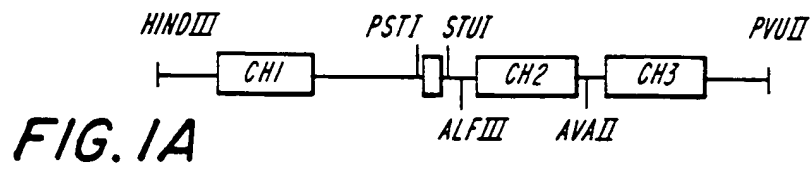
-33-

14. The antibody construct of claim 9 wherein said protein comprises a tumoricidal protein selected from the group consisting of epidermal growth factor, tumor necrosis factor, ricin, diphtheria toxin, 5 Psuedomona exotoxin, active analogs thereof, and active fragments thereof.

15. The construct of claim 1 further comprising a radioactive label.



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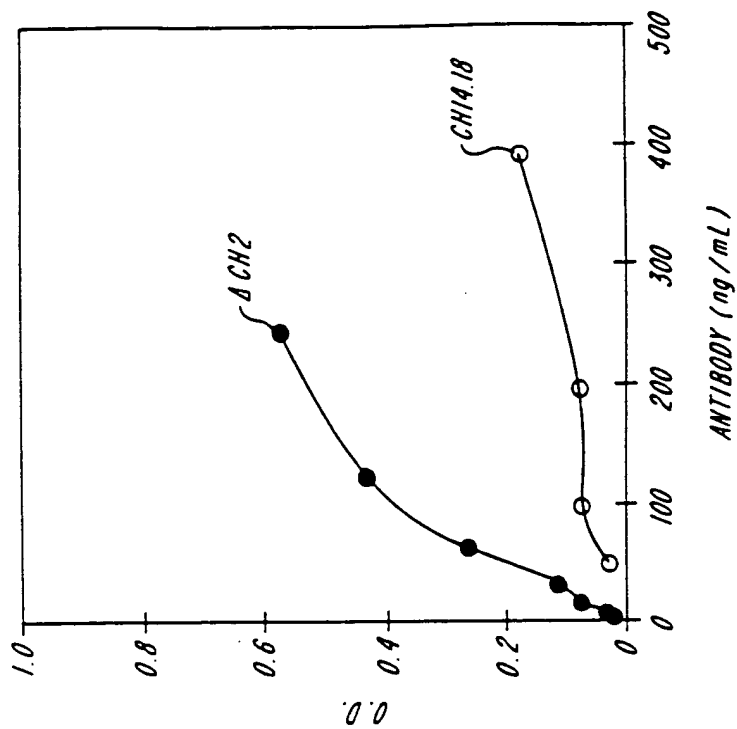


FIG. 2B

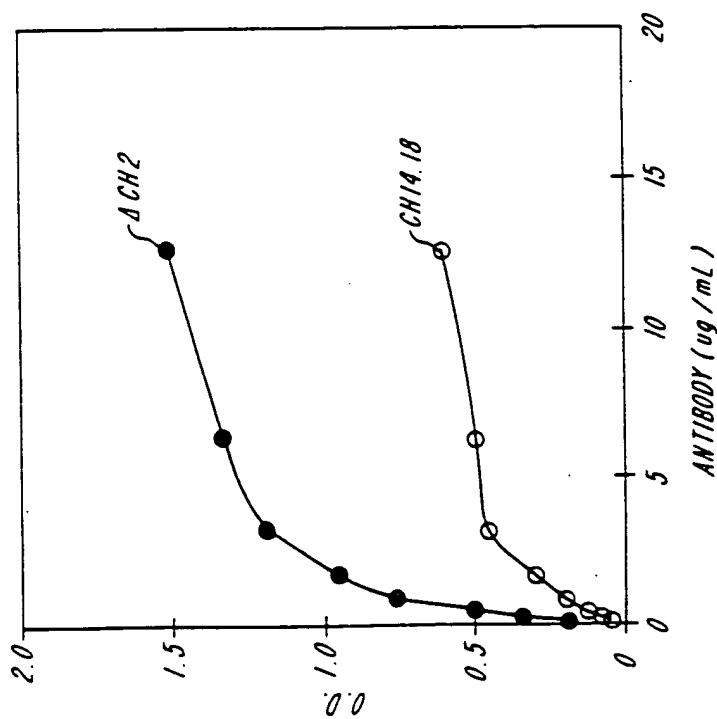


FIG. 2A





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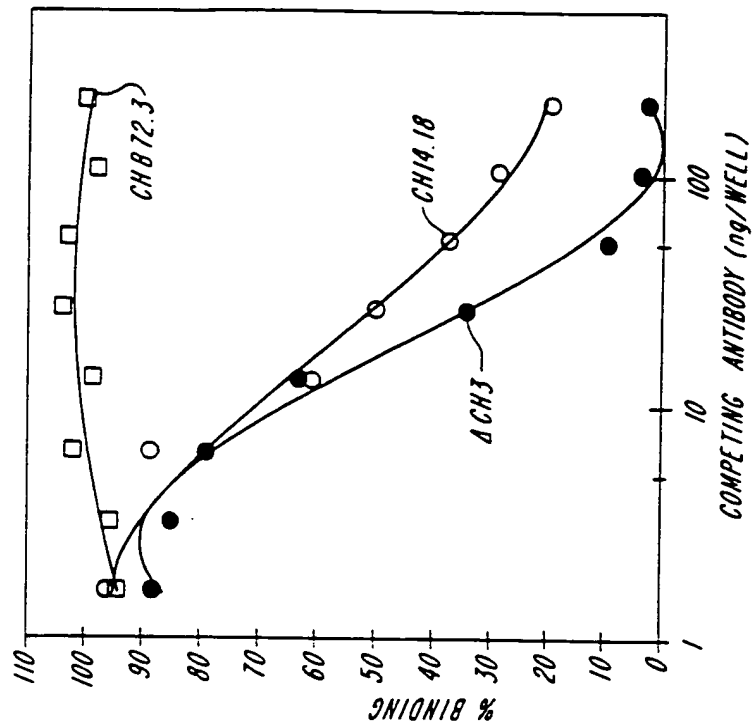


FIG. 3B

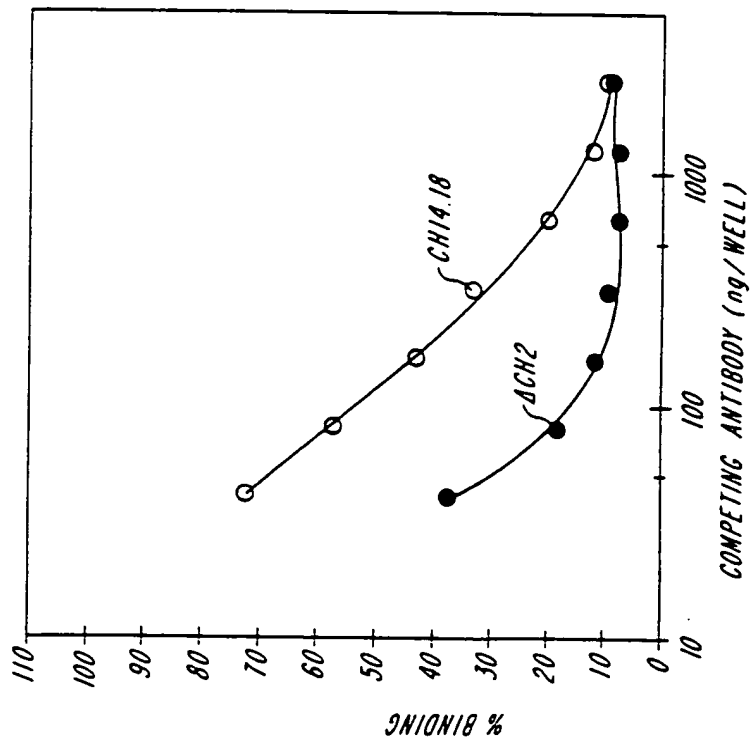


FIG. 3A



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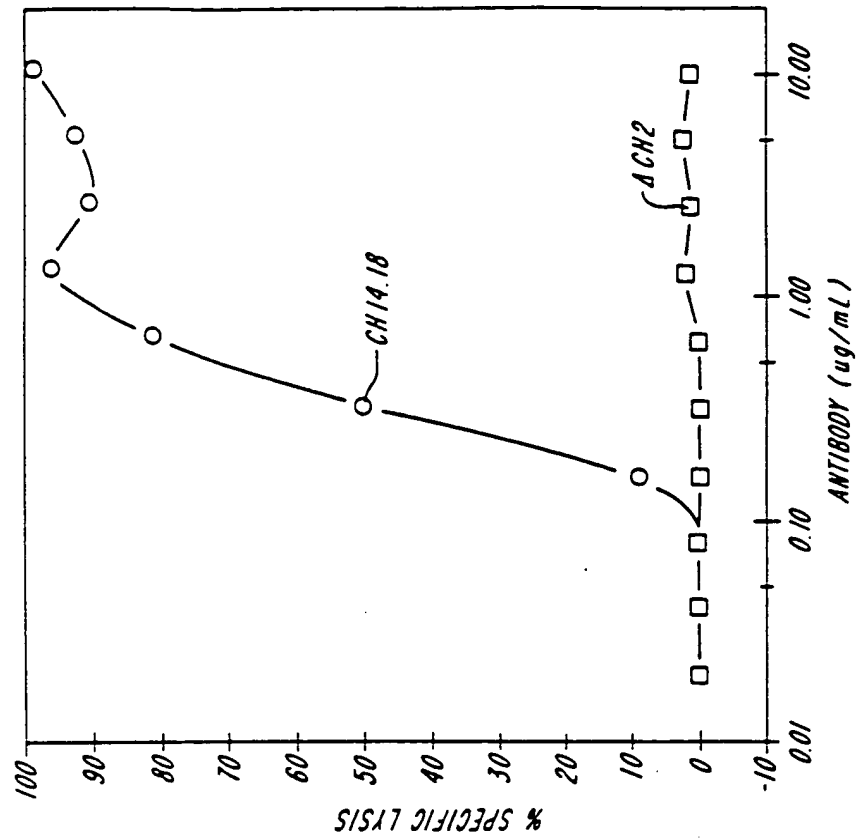


FIG. 5

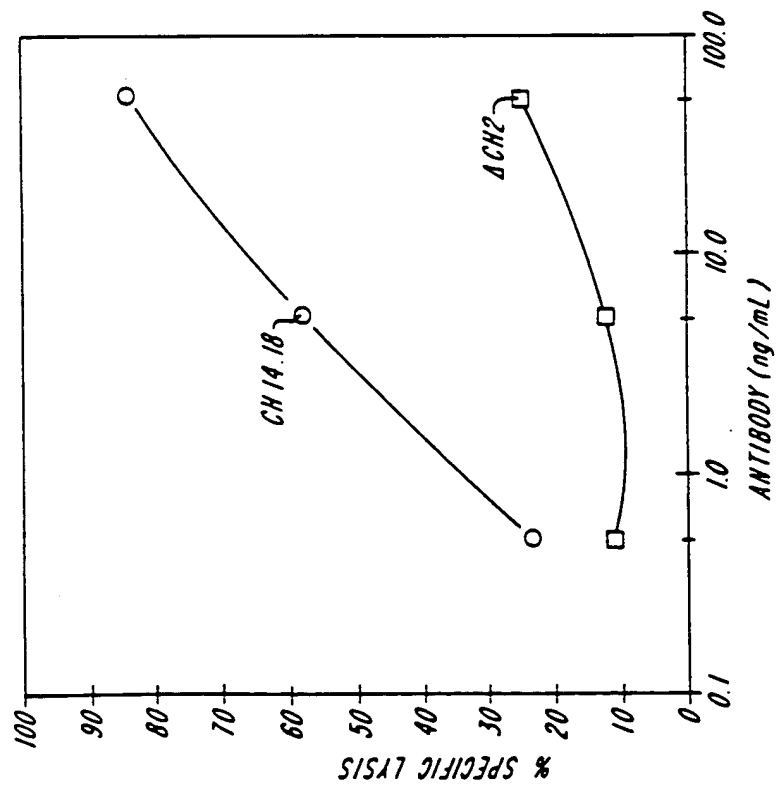


FIG. 4



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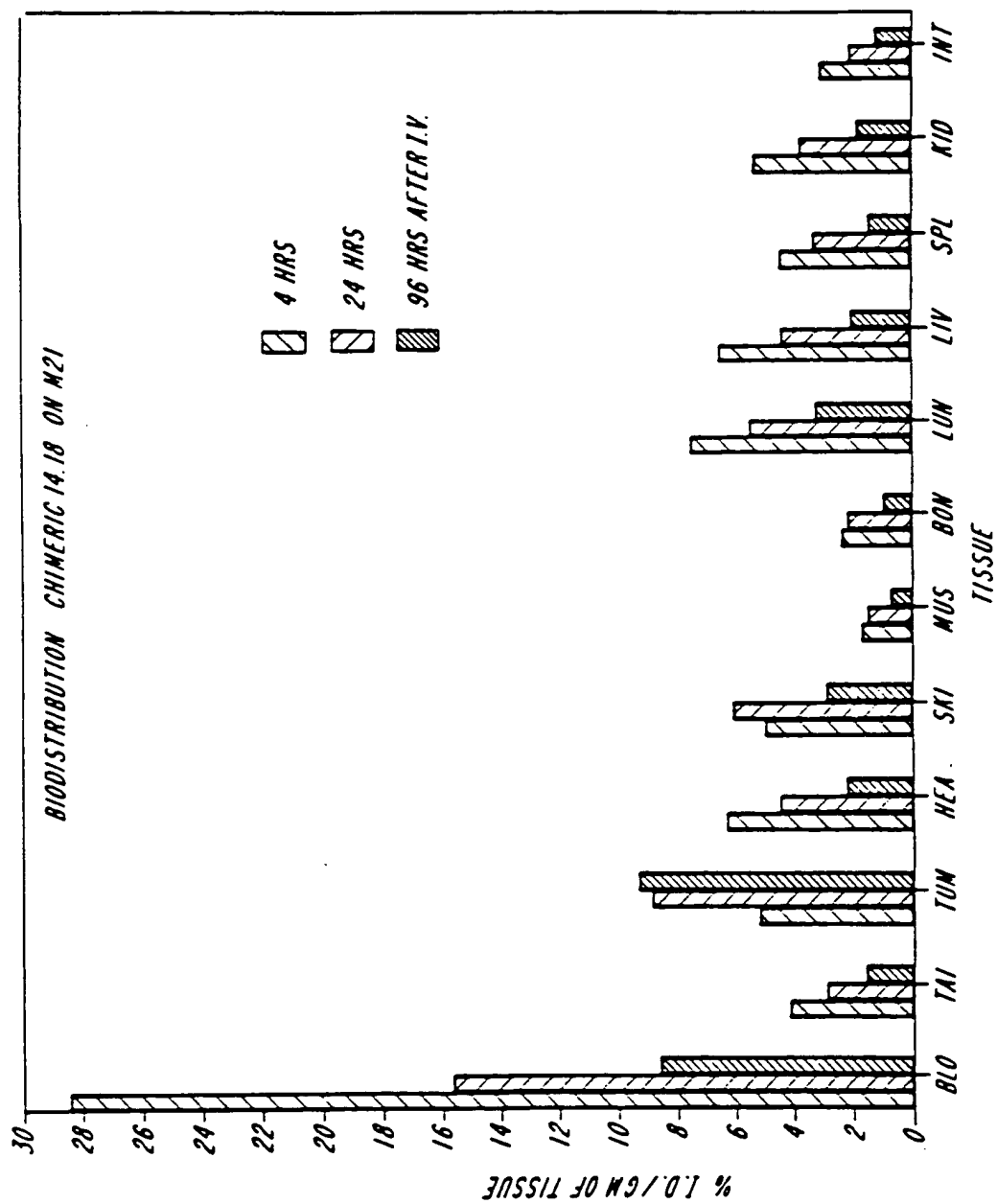


FIG. 6A



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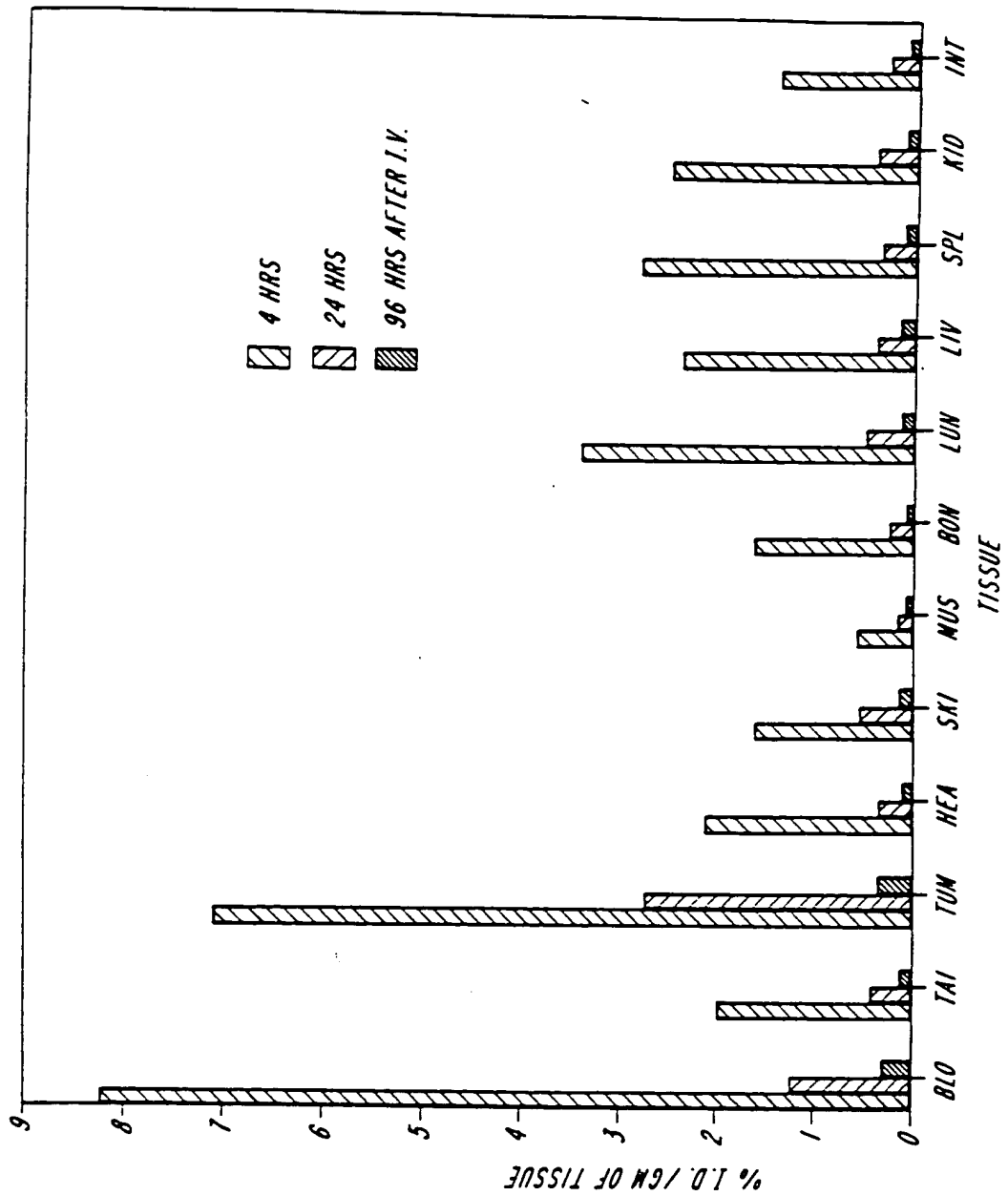


FIG. 6B





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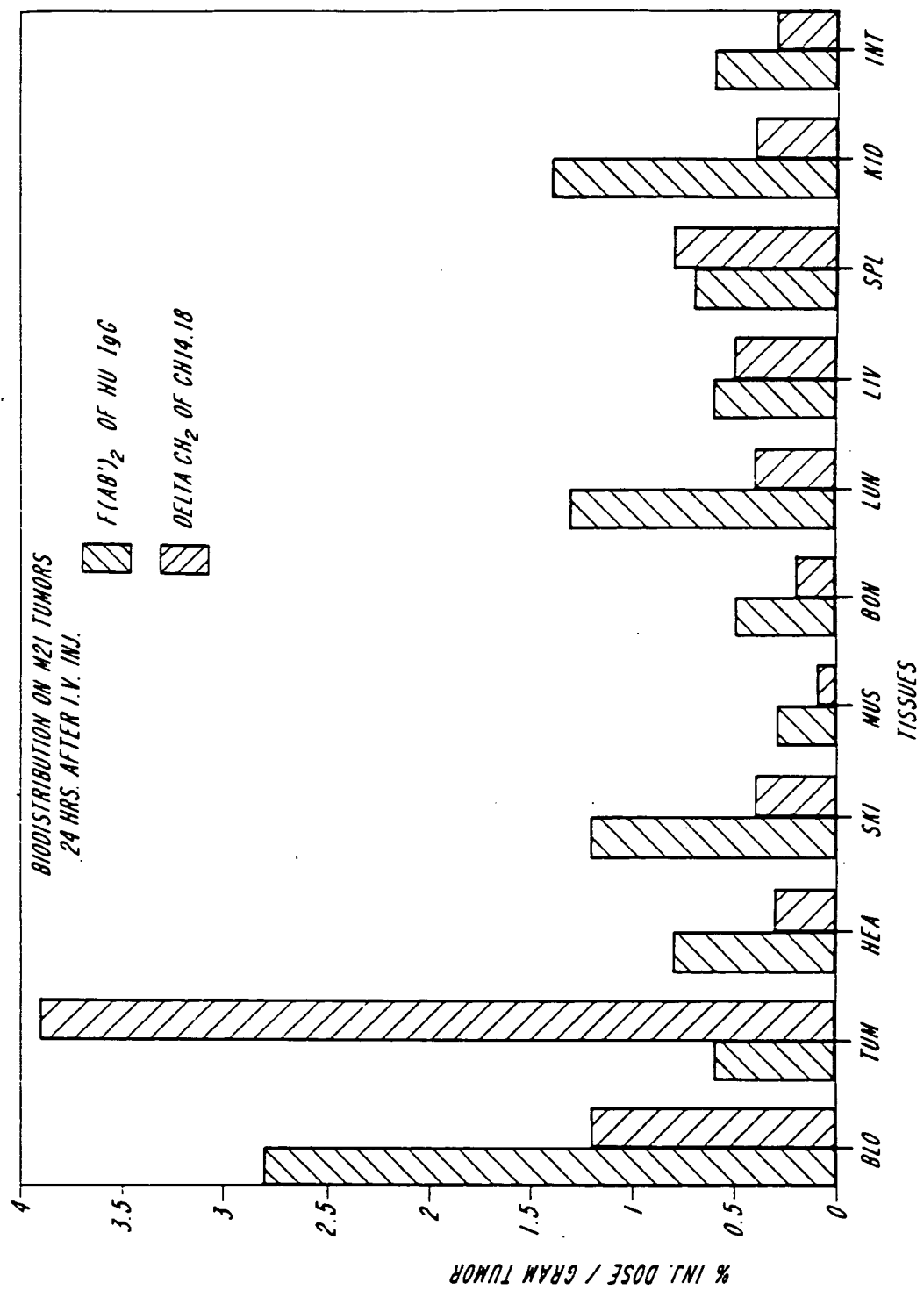


FIG. 7



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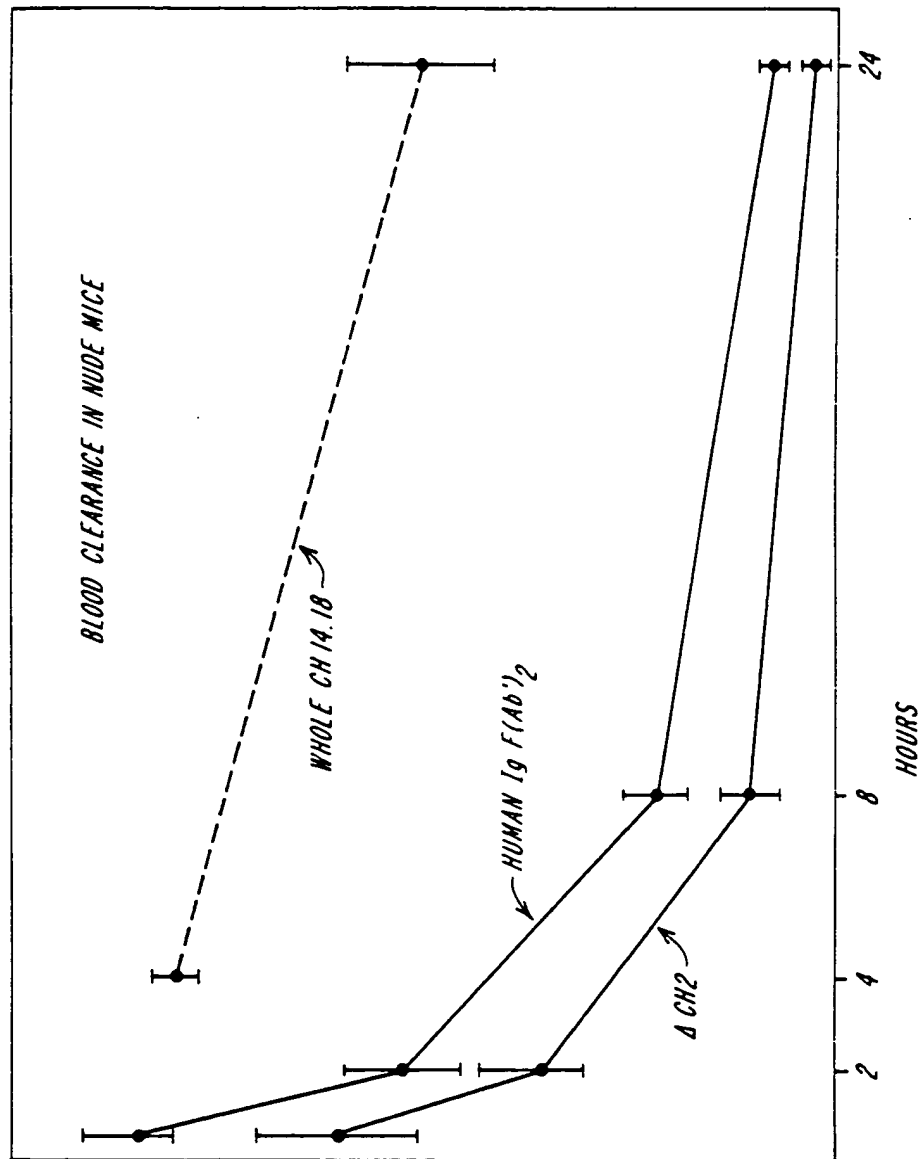


FIG. 8



# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 91/00633

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>5</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 12 P 21/00, C 07 K 15/00, A 61 K 39/395											
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <div style="display: flex; justify-content: space-between;"> <span>Classification System <sup>1</sup></span> <span>Classification Symbols</span> </div> <div style="margin-top: 10px;">           IPC<sup>5</sup> : C 12 P, C 07 K, A 61 K, C 12 N         </div> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup></div>											
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <thead> <tr> <th style="width: 10%;">Category <sup>10</sup></th> <th style="width: 70%;">Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">           EP, A2, 0 256 654            (CENTOCOR, INC.)            24 February 1988 (24.02.88),            see abstract.         </td> <td style="text-align: center; vertical-align: top;">1</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">           PROCEEDINGS OF THE NATIONAL            ACADEMY OF SCIENCES OF THE            UNITED STATES OF AMERICA,            vol. 85, no. 7, April 1988,            Baltimore, USA, R.R. POLLOCK            et al. "Identification of            mutant monoclonal antibodies            with increased antigen bin-            ding", pages 2298-2302, see            totality.         </td> <td style="text-align: center; vertical-align: top;">1</td> </tr> </tbody> </table>			Category <sup>10</sup>	Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	A	EP, A2, 0 256 654 (CENTOCOR, INC.) 24 February 1988 (24.02.88), see abstract.	1	A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 85, no. 7, April 1988, Baltimore, USA, R.R. POLLOCK et al. "Identification of mutant monoclonal antibodies with increased antigen bin- ding", pages 2298-2302, see totality.	1
Category <sup>10</sup>	Citation of Document <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>									
A	EP, A2, 0 256 654 (CENTOCOR, INC.) 24 February 1988 (24.02.88), see abstract.	1									
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 85, no. 7, April 1988, Baltimore, USA, R.R. POLLOCK et al. "Identification of mutant monoclonal antibodies with increased antigen bin- ding", pages 2298-2302, see totality.	1									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>14</sup> Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </div> </div>											
<b>IV. CERTIFICATION</b> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="text-align: center; margin-top: 10px;">17 May 1991</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center; margin-top: 10px;">02. 07. 91</div> </td> </tr> <tr> <td style="width: 50%; padding: 5px;">           International Searching Authority  <div style="text-align: center; margin-top: 10px;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center; margin-top: 10px;">Natalie Weinberg</div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; margin-top: 10px;">17 May 1991</div>	Date of Mailing of this International Search Report <div style="text-align: center; margin-top: 10px;">02. 07. 91</div>	International Searching Authority <div style="text-align: center; margin-top: 10px;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;">Natalie Weinberg</div>					
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ANHANG  
zum internationalen Recherchen-  
bericht über die internationale  
Patentanmeldung Nr.

ANNEX  
to the International Search  
Report to the International Patent  
Application No.

ANNEXE  
au rapport de recherche inter-  
national relatif à la demande de brevet  
international n°

PCT/US91/00633 SAE 45025

In diesem Anhang sind die Mitglieder  
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richtung und erfolgen ohne Gewähr.

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Veröffentlichung  
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date  
Date de  
publication

EP-A2- 256654

24-02-88

EP-A3- 256654  
JP-A2-63112995

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18-05-88